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LIGHT, a Member of the TNF Superfamily, Induces Morphological Changes and Delays Proliferation in the Human Rhabdomyosarcoma Cell Line RD

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LIGHT is a member of the tumor necrosis factor (TNF) superfamily, which binds two known receptors, lymphotoxin-β receptor (LTβR) and the herpesvirus entry mediator (HVEM)/TR2. We investigated the effects of LIGHT on the human rhabdmyosarcoma cell line RD. LIGHT delayed cell proliferation and induced morphological changes of the cells. These effects were not shown by other TNF family ligands such as TNFα and LTα, which induced the transcriptional activity of nuclear factor-κB (NF-κB) and NF-κB-responsible chemokine productions in the same manner as did LIGHT. LTα1β2, another TNF family ligand for LTβR, was shown to have similar activities in RD cells as LIGHT. Both LIGHT and LTα1β2 induced the expression of muscle-specific genes such as smooth muscle (SM) α-actin, while TNFα and LTα did not. These findings indicate that LIGHT may be a novel inducer of RD cell differentiation associated with SM α-actin expression through the LTβR. © 2001 Elsevier Science

Key Words: **LIGHT; RMS; RD; cell growth; differentiation; smooth muscle α-actin; TPA; LTβR.**

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children originating from immature cells and it has a long-term survival rate in children of only 50–70% (1, 2). These tumors resemble primitive skeletal muscle-forming cells in appearance and are highly aggressive, suggesting that RMS may arise from skeletal muscle cells that are arrested along the normal myogenic pathway to maturation (1, 2). Several studies of the molecular basis in RMS have shown RMS to be associated with loss of heterozygosity (LOH) at the 11p15 locus, which effects the expression of insulin-like growth factor (IGF), which is a growth factor of RMS (3, 4). RMS is characterized by the expression of several muscle-specific markers such as myogenic-promoting transcription factor MyoD. Although the expression of such factors typically correlates with myogenic differentiation, RMS fails to undergo terminal differentiation into skeletal muscle (2).

Members of the TNF family play important roles in cell activation, proliferation, differentiation, apoptosis, immunoglobulin class switch, immune evasion, and immune suppression (5–8). Recently, a new member of the TNF family, designated as LIGHT, was identified as a cellular ligand for both herpesvirus entry mediator (HVEM), also designated as TR2, and LTβR (9–13). LIGHT mRNA is highly expressed in splenocytes, activated PBL, $CD8^+$ tumor-infiltrating lymphocytes, granulocytes, and monocytes but not in the thymus or the tumor cells examined to date (9). LTβR is prominent on epithelial cells but absent in T and B lymphocytes (14); it is also involved in the development of peripheral lymph nodes and spleen architecture (15, 16). LTβR is the receptor for LIGHT as well as membrane-bound LTα1β2 trimers (10, 14), and HVEM/TR2 has been shown to be the receptor for LIGHT, LTα, and herpes simplex virus envelope glycoprotein D (17, 18). Recently, LIGHT was reported to be able to induce apoptosis in several tumor cells (9), and has a CD28-independent costimulatory activity leading to T-cell growth and differentiation (11). Thus, LIGHT is a pleiotropic molecule initiating diverse biological functions depending on the receptor expression profiles of the target cells.

We found that LIGHT delayed cell proliferation and induced morphological changes in a human RMS cell line, RD, associated with the expression of smooth muscle α-actin mRNA. LTα1β2, another TNF family ligand for LTβR, had similar effects on RD cells, but

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TNFα and LTα did not. Therefore, LIGHT may be a novel inducer of morphological changes on RMS by expressing cytoskeletal protein(s) through LTβR.

MATERIALS AND METHODS

Materials and cell culture. Human rhabdomyosarcoma cell line RD was purchased from the American Type Culture Collection (Rockville, MD). The cells were maintained in DMEM containing 10% fetal bovine serum and 1 mM sodium pyruvate. Recombinant human TNFα, LTα, and LTα1β2 were purchased from R&D Systems (Abingdon, UK). 12-*o*-Tetracanoylphorbol-13-acetate (TPA) was purchased from Wako Pure Chemical Industries (Osaka, Japan). RANTES and IL-8 productions were measured using Quantikine kits (R&D Systems). Soluble LIGHT proteins were produced and purified as follows: a full-length human LIGHT cDNA was obtained from a SUPERSCRIPT human liver cDNA library (Gibco-BRL, MD) using the GENETRAPPER cloning system (Gibco-BRL) with the following probes and colony-PCR primers: 5′-AGGTCAACCCAGCAGCGCATCTCA-3′ and 5′-CACCATCACCCACGGCCTCTACAAG-3′, for cDNA cloning, 5′-AGGTCAACCCAGCAGCGCATCTCACAGG-3′ and 5′-CAAATTAAACCGGGTACCATCACGCAGTCG-3′, for colony-PCR. The extracellular region (encoding Ile^{84} to Val^{240}) of LIGHT was amplified from the cDNA by PCR using the following primers: 5′-GAATTCGATACAAGAGCGAAGGTCTCACGAGGTC-3′ and 5′-AAATCTAGATCCTTCCTTCACACCATGAAAGCCCC-3′. The PCR product was digested with *Eco*RI and *Xba*I, and ligated into the *Eco*RI–*Xba*I site of pFLAG-CMV-1 expression vector (Eastman Chemical Company, NY). The preprotrypsin-FLAG-LIGHT DNA region in the vector was further digested with *Sac*I and *Xba*I, and ligated into the *Sac*I–*Xba*I site of pFAST-BAC1 vector (Gibco-BRL). The plasmid was infected into SF9 insect cells to generate the recombinant LIGHT proteins according to the procedure of Bac-to-Bac Baculovirus Expression System (Gibco-BRL). The FLAG-tagged soluble LIGHT proteins were purified with an anti-FLAG mAb affinity column, ANTI-FLAG M2-Agarose (Sigma, MO).

Semiquantitative RT-PCR analysis. Semiquantitative PCR amplification using primers for either human SM α-actin mRNA (5′-GCTCACGGAGGCACCCCTGAA-3′ and 5′-CTGATAGGACATTGTTAGCAT-3′), human β-actin mRNA (5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ and 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′), human myogenin mRNA (5′-CCGTGGGCGTGTAAGGTGTG-3′ and 5′-ACGATGGAGGTGAGGCAGTGC-3′), or human Id-1 mRNA (5′-CGAGGTGGTGCGCTGTCTGTCT-3′ and 5′-TCGCCGTTGAGGGTGCTGAG-3′) was performed using the Advantage 2 PCR Enzyme Systems (Clontech, CA) to amplify the 591-bp fragment for SM α-actin cDNA, 540-bp fragment for β-actin cDNA, 416-bp fragment for myogenin cDNA, or 315 bp fragment for Id-1 cDNA, respectively. We used 18S rRNA cDNA detection as an internal control.

Cell proliferation assay. Cell proliferation assays were performed using the cell proliferation ELISA, BrdU kit (Roche, NY). Briefly, after the RD cells (2500 cells/well) were cultured in 96-well plates with each ligand for 4 days, the cells were labeled by adding 5-bromo-2′-deoxyuridine (BrdU) solution for 1.5 h (final concentration of 10 μM), before being washed and fixed. The cells were treated with an anti-BrdU-POD antibody for 1.5 h. After washing, the cells were treated with POD substrate solution and the absorbance at 450 nm/690 nm was measured with a plate reader. In a separate procedure, the RD cells were plated in duplicate in 25 cm^2 flasks with each ligand for 6 days. The living cell number was determined using the trypan blue exclusion method.

Flow cytometry. RD cells (1×10^6 cells) were cultured with or without LIGHT for 72 h, fixed in 70% ethanol for 24 h at −20°C, and washed with PBS (−). The cells were incubated with 2 mg/ml RNase A for 20 min at 37°C, and stained with propidium iodide for 30 min at room temperature. The DNA content of the cells was determined using a flow cytometry FACScan (Becton–Dickinson, Germany).

NF-κB transcriptional activity assay. The NF-κB transcriptional activity was determined using a Mercury Pathway Profiling System (Clontech). Briefly, RD cells (1×10^6 cells) were seeded in a 12-well plate for 1 day and were then transfected with 0.5 μg pNF-κB-SEAP vector using 1.5 μl FuGENE6 reagent (Roche) for 20 h. After the cells were exposed with or without each ligand for the indicated times, the SEAP activity in the culture media was determined using a Great EscAPe chemiluminescence detection kit (Clontech).

Western blot and immunocytochemistry. Western blot analysis was performed using a ProtoBlotII AP System (Promega, Germany). Briefly, the RD cells (7×10^4 cells) were cultured with reagents for 6 days and lysed with a high salt buffer (0.6 M KCl in 10 mM Tris, pH 7.5, and protease inhibitors). The soluble proteins collected by centrifugation from the lysates were separated by 2–15% SDS–polyacrylamide gels, and then transferred onto nitrocellulose membranes. The membranes were then incubated with a mouse anti-skeletal myosin monoclonal antibody (mAb) MY-32 (Zymed, CA) at 1:100 dilutions for 1 h at room temperature. After washing, the blots were incubated for 1 h with an anti-mouse IgG(H + L) AP Conjugate (Promega) at 1:5000 dilutions before being exposed to the substrate solution. For immunocytochemistry, the RD cells (6×10^3 cells) were cultured with various reagents for 6 days and then fixed in ethanol:acetone (1:1) for 30 min at −20°C before being blocked with PBS(+) containing 1% BSA. The cells were then incubated with MY-32 mAb for 1 h, following HRP-anti-mouse IgG $F(ab)'_2$ (ICN/Cappel, OH) as the 2nd antibody against MY-32 mAbs. After washing, diaminobenzidine (Sigma, MO) was used as a peroxidase substrate to visualize the stained cells.

RESULTS

Growth Delay Induced by LIGHT

As shown in Fig. 1A, we found that LIGHT at a concentration above 6 ng/ml had a substantial growth inhibitory effect on a human embryonal rhabdomyosarcoma cell line RD. Though LIGHT inhibited the total viable RD cell number by up to about one-third during the first 6 days compared with the control growth (Fig. 1B), reexposure of the cells with an excess amount of LIGHT (up to 50 ng/ml) did not result in complete suppression of growth. Furthermore, when the cell cycle distribution of the LIGHT-treated cells was compared with that of control cells by flow cytometry, the G_0/G_1 percentage of the cells increased only slightly, from 49 to 60%, even after 6 days of treatment (Fig. 1C). Therefore, the effect of LIGHT on the RD cells seemed to involve a delay of cell proliferation rather than a frank arrest of the growth. A similar inhibitory effect was observed with LTα1β2, while the other TNF family ligands, such as TNFα and LTα, had only a weak suppressive effect on RD cell proliferation (20% inhibition according to the trypan blue exclusion method) (Fig. 1B). Interestingly, LTα synergistically stimulated the inhibitory effect of LTα1β2, as shown in Fig. 1.

Morphological Changes Caused by LIGHT

Observations with phase-contrast microscopy showed that the first 2 days of treatment with 50 ng/ml of LIGHT induced no morphological changes in the RD

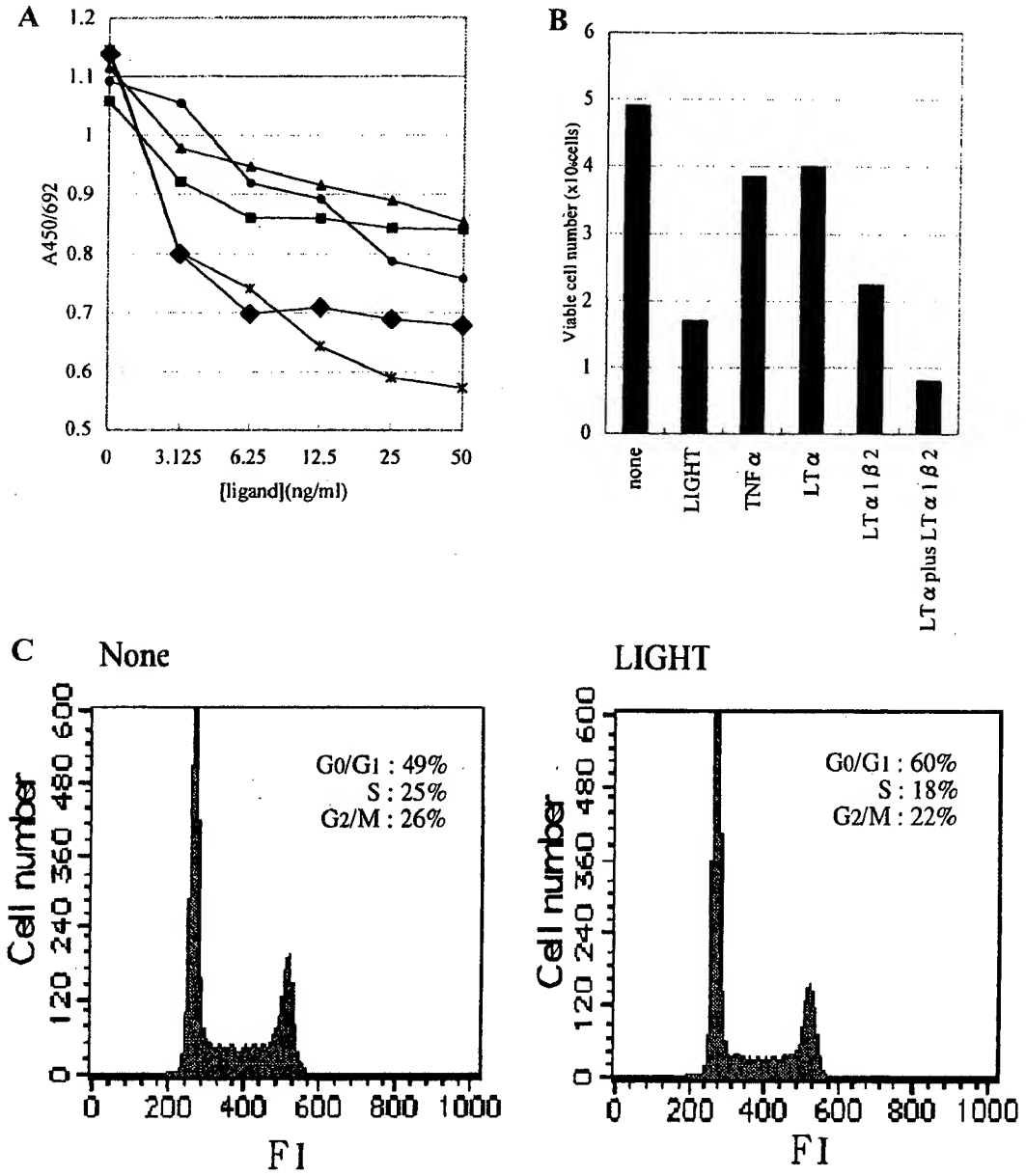

FIG. 1. Growth inhibitory activity of LIGHT on RD cells. (A) RD cells were treated with varying concentrations of LIGHT (◆), TNFα (■), LTα (▲), LTα1β2 (●), and LTα plus LTα1β2 (×) for 4 days, and OD 450 nm/690 nm was measured by a plate reader to determine the amount of BrdU incorporation. (B) RD cells (10^5 cells/well) were cultured for 6 days with 50 ng/ml of each ligand, and the viable cells in each well were counted after staining with trypan blue. (C) RD cells cultured with or without 50 ng/ml LIGHT for 3 days were stained with propidium iodide for 30 min at room temperature, and after establishing fractionated mononuclear cell populations, the DNA content of the cells was determined by flow cytometry. FI, fluorescence intensity.

cells compared with control cells (Fig. 2A). After the 5th day of culture, however, more than half of the cells treated with LIGHT showed an evident increase in elongated cytoplasm hypertrophy and formed multinucleated myotube-like cells (Fig. 2B). Cells treated with LTα1β2 showed similar morphological changes to those treated with LIGHT (Fig. 2C). LTα synergistically promoted the changes induced by LTα1β2, whereas LTα alone did not (Figs. 2D and 2E). As shown in Fig. 2F, 12-*o*-tetracanoylphorbol-13-acetate (TPA), a known inducer of RD cell differentiation (19, 20), induced a different overall morphology that seen in the control or LIGHT-treated cells. This suggests that LIGHT causes a qualitatively different induction of changes in the morphology of RD cells than does TPA.

NF-κB Activation and Chemokine Productions

To examined whether LIGHT regulates the transcriptional activity of NF-κB in the manner of other

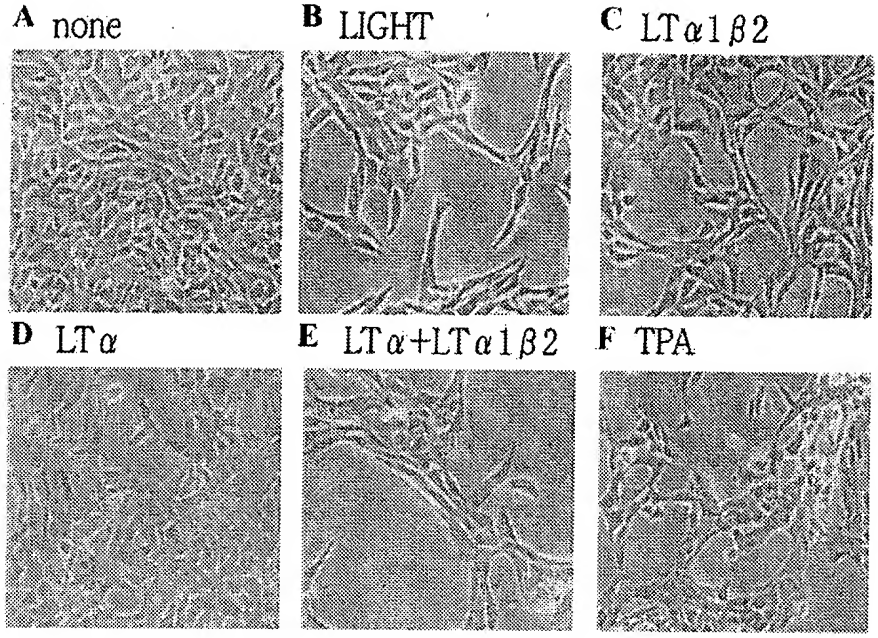

FIG. 2. Morphology of each of the samples of ligand-treated RD cells. Each phase-contrast image represents control RD cells (A), RD cells treated with 50 ng/ml LIGHT (B), LTα1β2 (C), LTα (D), LTα plus LTα1β2 (E), or 100 μg/ml TPA (F) for 5 days. All images were acquired at the same magnification using a ×10 objective lens.

ligands, we monitored the capacity of each ligand that induced the expression of representative NF-κB-responsive proteins, such as IL-8 or RANTES in RD cells. As shown in Fig. 3, all ligands induced comparative biological responses including IL-8 and RANTES productions in the cells cultured for 3 days. The productivity of each chemokine by LIGHT was similar to that by LTα1β2. Further experiments were performed to determine whether this responsiveness was related to the transcriptional activity of NF-κB. We used a secreted alkaline phosphatase (SEAP) reporter gene construct driven by four tandem copies of Kappa (κ) enhancer element (κB4;8) as an NF-κB responsive sequence in the Mercury Pathway Profiling Systems (Clontech), as described in materials and methods, and found that all ligands induced comparative levels of SEAP in RD cells. By contrast, the activity of NF-κB induced by LIGHT was weaker than that induced by other ligands (Fig. 4). Thus, even though engagement of TNFα or LTα induces normal transcriptional activity of NF-κB in RD cells, the activated NF-κB was not able to transactivate the growth delay or morphological conversions of RD cells.

Induction of Smooth Muscle α-Actin Gene Expression

To investigate possible roles of LIGHT in regulating morphological changes of RD cells, we examined apparent alterations of muscle regulatory genes and their protein products. By semiquantitative RT-PCR, we analyzed the accumulation of muscle-specific gene transcripts such as smooth muscle (SM) α-actin, myogenin, and Id-1. LIGHT and LTα1β2 induced expression of SM α-actin mRNA, whereas other ligands such as TNFα or LTα did not. The expression of β-actin, myogenin, and Id-1 were not changed in LIGHT-treated RD cells (Fig. 5). It has been reported that RD cells treated with TPA increase their expression of cytoskeletal proteins such as skeletal muscle myosin and skeletal muscle α-actin, increase their binding of ^{125}I-α-bungarotoxin, and increase the phosphorylation of several proteins including α-PKC (19, 20). While TPA did not modify the expression of SM α-actin or myogenin in the present study, it induced skeletal myosin expression in RD cells according to a combined analysis using both immunocytochemistry (Fig. 6A) and Western blot analysis (Fig. 6B) with an anti-skeletal muscle myosin mAb MY-32. Conversely, no positive cells or skeletal myosin proteins were found in RD cells treated with LIGHT according to this analysis (Figs. 6A and 6B). Immunocytochemistry and Western blot analysis using an anti-smooth muscle myosin heavy chain mAb F126.16D9 (Biocytex, France) showed that none of the ligands tested changed the expression level of smooth muscle myosin proteins (data not shown).

DISCUSSION

LIGHT is a member of the TNF superfamily, which binds two known receptors, LTβR and HVEM/TR2. Our results indicated that LIGHT plays an important role in the differentiation process of human rhabdomyosarcoma cell line RD. LIGHT caused a marked

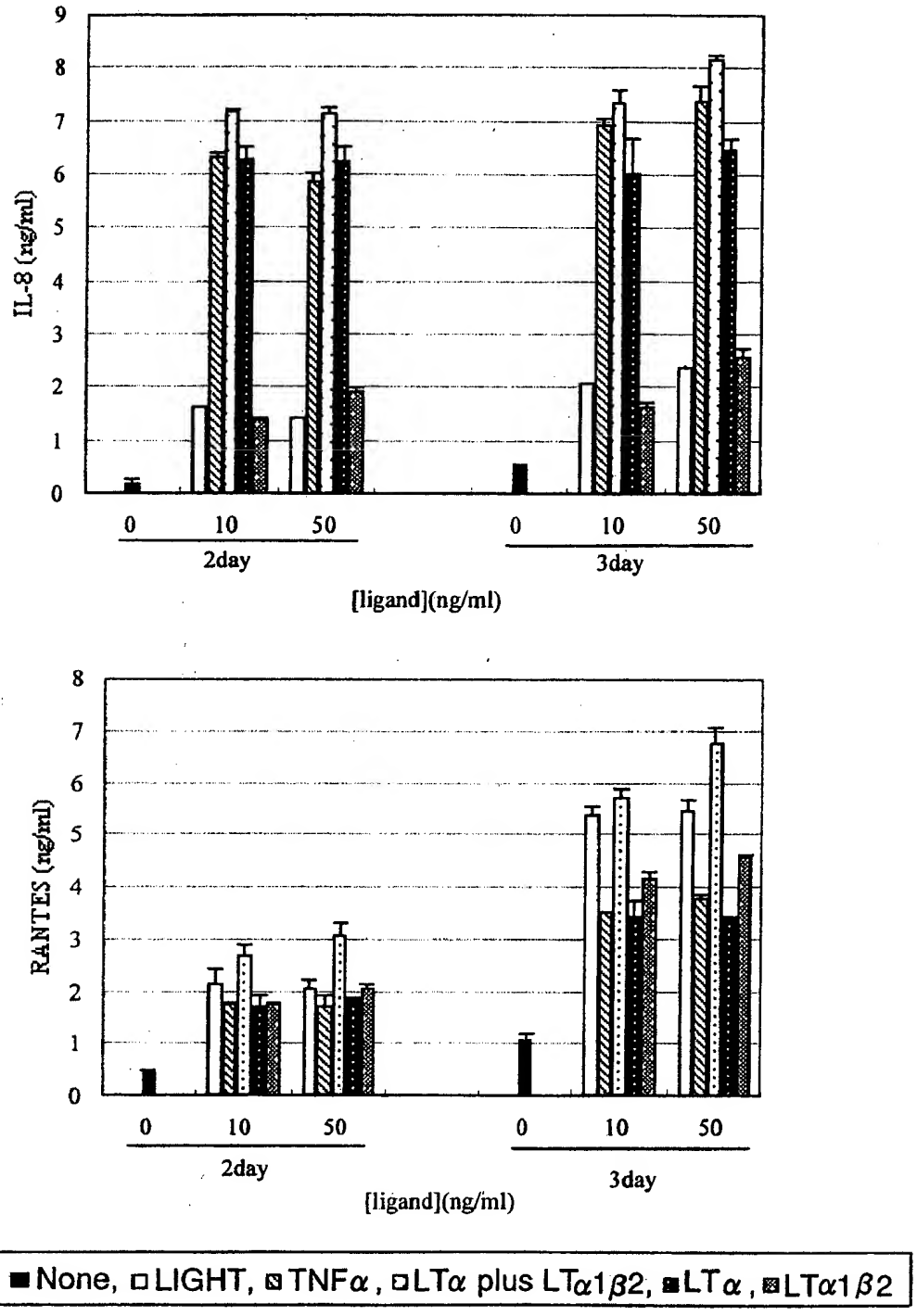

FIG. 3. Chemokine production of RD cells by LIGHT. RD cells were cultured with the indicated concentrations of each ligand for 2 and 3 days. IL-8 and RANTES were measured by each specific ELISA kit.

morphological change in the RD cells characterized by growth delay with elongated cytoplasm hypertrophy (Figs. 1 and 2) and increased smooth muscle (SM) α-actin expression (Fig. 5). Furthermore, our results demonstrated that such changes in the RD cells are not caused by TNFα or LTα, except for LTα1β2, which is another TNF family ligand specifically bound to LTβR. Both TNFα and LTα stimulated the activation of NF-κB and the production of NF-κB-responsive chemokine in the manner of both LIGHT and LTα1β2, indicating that LTβR signaling may directly activate differentiation pathways in RD cells (Figs. 3 and 4).

RD is an embryonal RMS resembling normal fetal skeletal muscle in morphology and it expresses several

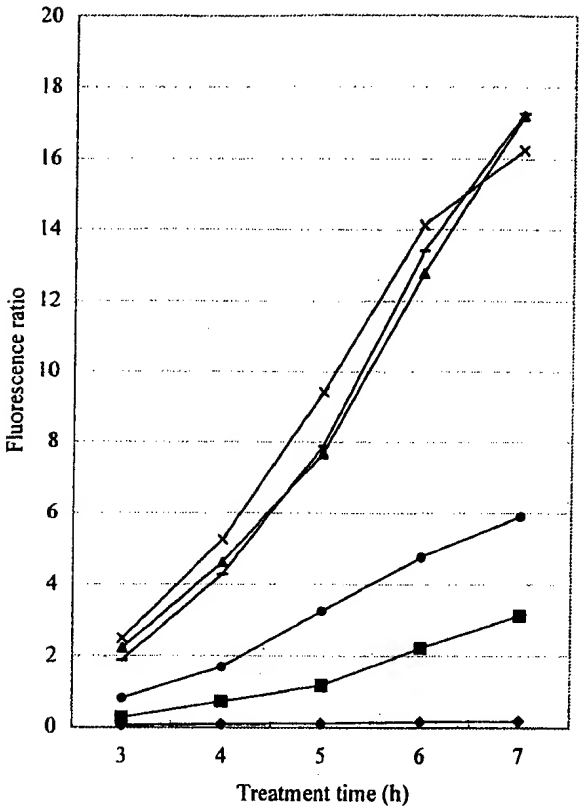

FIG. 4. NF-κB transcriptional activity of LIGHT on RD cells. RD cells transfected with 0.5 mg of pNF-κB-SEAP vector (Clontech) were cultured with 50 ng/ml LIGHT (■), LTα1β2 (●), TNFα (×), LTα (−), LTα plus LTα1β2 (▲), or the control (◆) for the indicated times. After the treatment, the SEAP activity in culture media was determined using a Great EscAPe chemiluminescence detection kit (Clontech).

muscle-specific genes such as the myogenic-promoting transcription factor MyoD. RD cells are capable of only a limited and abortive spontaneous myogenic differentiation, probably because they lack functional p53 (21) and have homozygous gene deletion of $p16^{ink4}$ gene (22). Recent evidence suggests that transfection with a temperature-sensitive p16 mutant (E119G) gene in RD cells, under a permissive culture condition, reduced CDK6-associated kinase activity, induced G1 growth arrest, and induced morphological change coupled with the expression of myogenin and myosin light chain genes (22). However, Knudsen *et al.* reported that ectopic expression of $p21^{cip1}$, $p16^{ink4}$, or $p27^{kip1}$ in RD cells caused the cell growth arrest, but not detectable expression of myogenic markers such as myosin heavy chain, indicating that these activities alone are not sufficient for RD cells to differentiate (23). In any case, the failure of RMS to undergo terminal differentiation into skeletal muscle may be one mechanism by which these cells gain the growth advantage necessary for tumor formation. While the growth inhibitory effect of LIGHT on RD cells was obvious at a concentration of 6 ng/ml (Fig. 1A), at 50 ng/ml LIGHT, which is an excessive amount, the inhibition increased only marginally, suppressing the cell number by one-third compared with the control (Fig. 1B). When we compared the cell cycle distribution in LIGHT-treated cells with that for control cells, the G_0/G_1 phase percentage was only slightly increased, from 49% to 60% (Fig. 1C). The flow cytometry analysis was performed after excluding the differentiated cells with elongated cytoplasm hypertrophy and multinucleated myotube-like cells. Thus, there may be the possibility that the differentiated cells preferentially arrest cell growth in the culture system. Therefore, the reduction in cell number by LIGHT may be due to morphological conversion of part of the RD cells rather than a typical growth arrest or cytotoxicity. Since we did not investigate the growth characteristics of the morphologically changed cells, more experiments are needed to better understand these phenomena.

TPA is known to be a differentiation reagent of RD cells accompanied with inducible expression of muscle-specific genes such as skeletal muscle α-actin and myosin light chain genes. Both LIGHT- and LTα1β2-treated RD cells markedly expressed SM α-actin gene (Fig. 5), but TPA did not. By contrast, TPA slightly reduced the expression of Id-1 mRNA, a negative reg-

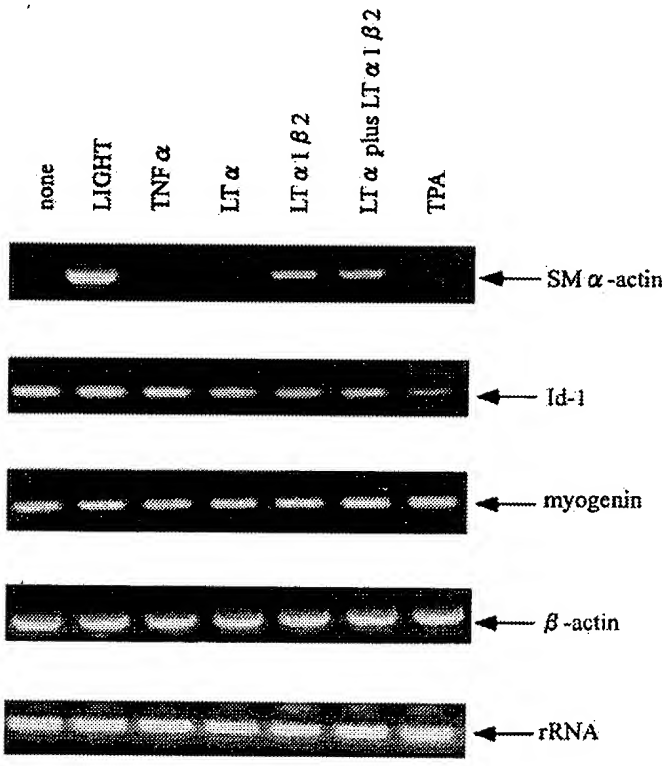

FIG. 5. Expression of muscle-specific genes in RD cells by LIGHT. After the RD cells were cultured with 50 ng/ml of each ligand or 100 μg/ml of TPA for 6 days, RT-PCR was performed on each sample of cells using primers specific for SM α-actin, Id-1, myogenin, β-actin, and rRNA. The PCR products were resolved on 1% agarose gel containing ethidium bromide to stain the DNA.

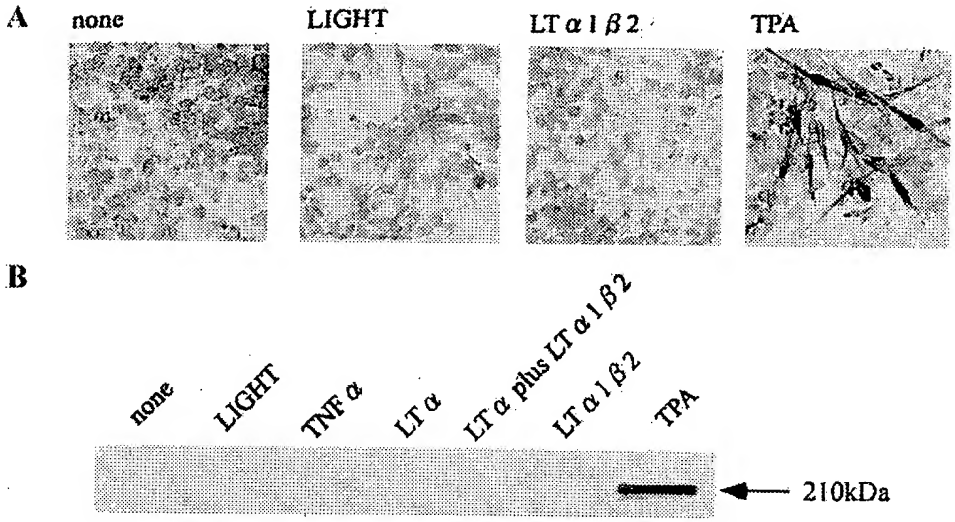

FIG. 6. Expression of skeletal muscle-specific myosin heavy chain proteins in TPA- and LIGHT-treated RD cells. (A) RD cells plated onto coverslips were cultured with or without 50 ng/ml of each ligand or 100 μg/ml of TPA for 6 days. After being fixed, each treated sample of cells was stained with an anti-skeletal muscle-specific myosin heavy chain mAb MY-32. (B) For Western blot analysis, each ligand-treated sample of cells was cultured for 6 days and lysed, and for each a 50 μg protein extract was used for Western blot analysis with MY-32 mAb.

ulator of MyoD, but LIGHT and LTα1β2 did not (Fig. 5). Conversely, skeletal myosin expression was increased in TPA-treated RD cells, but not in those treated by LIGHT or LTα1β2 (Fig. 6). These results suggest that the differentiation state induced by LIGHT and LTα1β2 is completely different from that induced by TPA. The SM α-actin gene is well-known to be activated during the early stage of embryonic cardiovascular development, switched off in late stage heart tissue, and replaced by cardiac and skeletal α-actins. It also appears during vascular development, and becomes the most abundant protein in adult vascular smooth muscle cells. Tissue-specific expression of SM α-actin is required for the principal force-generating capacity of the vascular smooth muscle cells. Therefore, LIGHT might activate the transcriptional machinery necessary for transdifferentiation from a skeletal- to a smooth-muscle lineage through LTβR.

Signaling by TNF family members is initiated by an aggregation of specific cell surface receptors. TNFα, LTα, LTα1β2, and LIGHT exhibit distinct but overlapping patterns of binding to four cognate receptors: TNF receptor type 1 (TNFR1), TNF receptor type 2 (TNFR2), LTβR, and HVEM/TR2, which together define a core group within the larger TNF superfamily. TNFα binds two receptors, TNFR1 and TNFR2, and LTα binds TNFR1, TNFR2, and HVEM/TR2. LTα1β2, predominantly expresses in activated T cells and specifically binds LTβR. Although LIGHT binds both LTβR and HVEM/TR2, the cross-utilization of the receptors suggests functional redundancy of the ligand. When we examined the expression levels of these four receptors via RT-PCR, we observed that they all expressed in the cells. However, the expression level of each receptor mRNA was quite different; the lowest being HVEM/TR2 (40 copies/ng total RNA), with the expression levels of LTβR, TNFR1, and TNFR2 being 125-, 215-, and 45-fold higher than that of HVEM/TR2, respectively (data not shown). Rooney *et al.* suggested that LTβR is necessary and sufficient for LIGHT-mediated apoptosis in a human adenocarcinoma cell line HT29 (13). However, Zhai *et al.* reported that both LTβR and HVEM/TR2 are involved cooperatively in the LIGHT-mediated killing of tumor cells, including HT29 cells (9). Furthermore, it has been reported that only the activation of LTβR by cross-linking with an anti-LTβR mAb could induce growth arrest and chemokine production in A375 melanoma cells (24), although we did not observe these effects in the cells by LIGHT or LTα1β2. This discrepancy might reflect a different ligand sensitivity for the cell line we used. Since we did not determine whether the function of HVEM/TR2 is sufficient for ligand-mediated signal activation on RD cells, the possibility of the phenotype conversion through the HVEM/TR2 needs to be studied in more detail.

RMS is the most frequent soft tissue malignancy in pediatric patients. It is known that several distinct histological subtypes of RMS have been described: alveolar, embryonal, botryoid, and undifferentiated (1, 2). Although we examined additional RMS cell lines, Hs729, A673, and A-204, to confirm whether LIGHT can also induce differentiation in these cell lines, we did not observe any effects in them (data not shown). Therefore, there might be certain RMS cell types which have the sensitivity to LIGHT. Several studies using RD cells have been attempted to regress the phenotype by evaluating agents that alter cellular growth as well as differentiation both in *in vitro* and *in vivo*. Pyrimidine analogues such as GR-891 (25) and Ara-C (1-α-D-arabino-furanosyl cytosine) (26) are reported to induce

growth inhibition and to stimulate the differentiation processes and terminal myogenic differentiation of RD cells correlating with several differentiation markers. The present study may be the first to report morphological changes in RD cells induced by the proteinaceous reagents LIGHT and LTα1β2. It is hoped that further investigation of these ligands and their potential role could lead to a novel and useful therapeutic approach for RMS.

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